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Mary E. (Molly) Ceperley
Primary Examiner Art Unit 1641
(703) 308-4239
Office: CM1-8D15
Mailbox: CM1-7E12

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ANTI-IDIOTYPE MONOCLONAL ANTIBODIES AGAINST ANTI-MICROCYSTIN ANTIBODY AND THEIR USE IN ENZYME IMMUNOASSAY

TOMOAKI TSUTSUMI,[1,2] SATOSHI NAGATA,[2] FUYUKO YOSHIDA[2] and YOSHIO UENO[1,2]*

[1]Department of Toxicology and Microbial Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, Ichigaya, Shinjuku-ku, Tokyo 162, Japan; and [2]Research Institute for Biosciences, Science University of Tokyo, Noda-shi, Chiba 278, Japan

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Tomoaki Tsutsumi, Satoshi Nagata, Fuyuko Yoshida and Yoshio Ueno. Anti-idiotype monoclonal antibodies against anti-microcystin antibody and their use in enzyme immunoassay. *Toxicon* **36,** 235–245, 1997.—Microcystins (MCs), a group of heptapeptide hepatotoxins produced by cyanobacteria, are suspected as tumor-promoter contaminants of environmental water. We have previously developed an enzyme-linked immunosorbent assay (ELISA) for MCs based on an anti-MC MAb (MAb-mc). We describe here the production of anti-idiotype MAbs (MAb-ids) which react with MAb-mc and their use in a new ELISA for MCs. For the production of MAb-id, hybridoma cells were generated from mice immunized with MAb-mc. Two MAbs were selected for their ability to inhibit the binding of MAb-mc to microcystin-LR (MCLR)-bovine serum albumin conjugate in ELISA. The one with the higher inhibitory activity, designated Id7 (IgG_1, κ), was further characterized. ELISA and immunoprecipitation analysis revealed that Id7 specifically bound to MAb-mc but not to control IgG_1, and the binding was inhibited by free MCLR. Therefore, Id7 is a MAb-id to MAb-mc and potentially possesses the structural image of MCLR. To establish MAb-id based ELISA, Id7 was tested for use in three types of competitive ELISA for MCs. The best format enabled reliable measurements of MCLR in the range of 100–1000 pg/ml with a coefficient of variation of less than 3%. In addition, microcystin-RR and microcystin-YR, principal MCs found in environmental water, were cross-reacted well (67–111% of MCLR) in the ELISA although 6(Z)-MCLR, a minor component, was less reactive (7% of MCLR). A comparative study of the MAb-id based ELISA with previously established MAb-mc based ELISA revealed good correlation ($n = 14$, $r = 0.97$) between the two methods for measurements of MCs content in freshwater samples. Thus, developed MAb-id based ELISA is an useful alternative for environmental monitoring of MCs.

*Author to whom correspondence should be addressed.

INTRODUCTION

Toxigenic species of cyanobacteria, such as *Microcystis aeruginosa*, commonly form water blooms in lakes and ponds in many regions of the world. These blooms sometimes intoxicate humans and animals. Microcystins (MCs), a group of hepatotoxins produced by cyanobacteria, are considered a primary cause of such intoxications (Carmichael and Falconer, 1993; Carmichael, 1996). The findings that MCs possess a hepatotoxicity, an inhibitory activity for protein phosphatase 1 (PP1) and 2A (PP2A) (Honkanen *et al.*, 1990; Yoshizawa *et al.*, 1990), and a tumor-promoting activity in mice and rats (Falconer, 1991; Nishiwaki-Matsushima *et al.*, 1992), suggest a connection between the intake of MCs and the incidence of primary liver cancer, as reported in Fusui and Haimen counties in China (Yu, 1995; Ueno *et al.*, 1996a). At present, more than 50 MCs have been chemically identified and they share the common cyclic heptapeptide structure including two variable L-amino acids and a unique amino acid, 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Harada, 1996). Adda appears to be of importance in the toxicity (Harada *et al.*, 1990; Nishiwaki-Matsushima *et al.*, 1991; Stotts *et al.*, 1993).

Since MCs are hazardous to humans and animals, their levels in environmental water must be monitored. Since the level of MCs in water is very low, physicochemical methods are hard to apply for mass monitoring of MCs. One suitable method for monitoring of MCs in environmental water is an immunoassay using anti-MC antibodies. Enzyme-linked immunosorbent assays (ELISAs) using polyclonal antibodies to MCs have been reported for environmental samples (Chu *et al.*, 1990; McDermott *et al.*, 1995). Recently, we have produced an anti-microcystin-LR (MCLR) monoclonal antibody (MAb-mc) (Nagata *et al.*, 1995) and developed a highly sensitive ELISA applicable for direct determination of MCs in freshwater (Nagata *et al.*, 1997). Application of this MAb-mc based ELISA has demonstrated the occurrence of MCs in various water sources worldwide (Ueno *et al.*, 1996b). In MAb-mc based ELISA, MAb-mc reacts to MCLR conjugated with bovine serum albumin (MCLR–BSA) coated on microtiter plates in the presence of sample containing free MCs. MCs in the samples inhibit the binding of MAb-mc to MCLR–BSA depending on their concentrations. Anti-idiotype MAb (MAb-id) against MAb-mc is thought to possess the structural image of MCLR and therefore is a possible surrogate of MCLR–BSA. Thus, MAb-id against MAb-mc is an alternative immunological reagent that may improve the assay performance.

MAb-ids for small molecules have been produced and applied for their ELISA, such as hormones (Altamirano-Bustamante *et al.*, 1991; Becker *et al.*, 1996) and phytotoxin (Shestowsky *et al.*, 1992). Quite recently, Liu *et al.* (1996) reported the production of polyclonal anti-idiotype antibodies against anti-MC antibodies but they failed to use it in ELISA for MCs.

We describe here the production of MAb-ids against MAb-mc, and use it in a new ELISA for MCs in freshwater. A part of the present date was reported as a preliminary form (Tsutsumi *et al.*, 1997).

MATERIALS AND METHODS

Materials

MCLR was isolated from broom samples collected from Lake Suwa, Japan according to a method previously reported (Harada *et al.*, 1988). Microcystin–RR (MCRR), microcystin–YR (MCYR) and 6(Z)-Adda

microcystin–LR (6(Z)-MCLR) were kindly provided by Dr. Ken-ichi Harada, Meijo University, Japan. The purities of these MCs were estimated at over 90% by HPLC analysis.

MCLR–BSA was prepared by conjugation of MCLR with BSA(Sigma, St Louis) using 1-ethyl-3,3′-dimethylaminopropyl-carbodiimide (PIERCE, Rockford). The molar ratio of MCLR to BSA was 1.3, as determined previously (Nagata *et al.*, 1995).

Gelatin, 3,3′,5,5′-tetramethylbenzidine (TMBZ), Tween 20 and 30% hydrogen peroxide were from Wako Pure Chemical (Osaka) Alkaline phosphatase-labeled streptavidin (ALP-SA) and horseradish peroxidase-labeled streptavidin (HRP-SA) were from Vector Laboratories (Burlingame).

Monoclonal antibodies

MAb-mc (M8H5), IgG_1 subclass, was purified as described (Nagata *et al.*, 1995). To produce Fab fragments of MAb-mc, MAb-mc was partially digested with immobilized papain (PIERCE, Rockford) according to the manufacture's instruction. Two IgG_1 MAbs, 5E11 (anti-vesicular stomatitis virus glycoprotein) (Nagata *et al.*, 1992) and OTA.7 (anti-ochratoxin A) (Kawamura *et al.*, 1989) were used for negative controls in some experiments. MAb-mc, its Fab fragments and 5E11 were biotinylated with NHS-LC-biotin (PIERCE, Rockford) as described (Nagata *et al.*, 1992). MAb-mc and OTA. 7 were coupled to Affi-Gel 10 (Bio-rad Laboratories, Hercules) as described (Kondo *et al.*, 1996).

Production of MAb-id

Female BALB/c mice (10 weeks of age) were subcutaneously immunized with 100 μg of purified MAb-mc once with complete Freund's adjuvant and once with incomplete Freund's adjuvant. Additional i.p. injections of 100 to 150 μg of MAb-mc without adjuvant were given a total of 8 times at intervals of 2 weeks. Three days after the last injection, the spleen cells were fused with Sp2/O mouse myeloma cells (Harlow and Lane, 1988). Culture supernatants of growing hybridoma were screened for MAb-id as described below.

Positive hybridomas were cloned by limiting dilution and expanded for large scale production of MAbs. Antibody purification, isotype determination and biotinylation were done as described previously (Nagata *et al.*, 1991, 1992).

Screening ELISA for MAb-id

The MAb-ids were screened for their ability to inhibit the binding of MAb-mc to coated MCLR–BSA in an ELISA. Microtiter plates (Coaster, Cambridge) were coated with MCLR–BSA (100 ng/ml in phosphate-buffered saline (PBS)) overnight at 4°C, then blocked with blocking buffer (0.5% (w/v) gelatin in PBS). Biotinylated Fab fragments of MAb-mc (200 ng/ml in blocking buffer) were incubated with equal volumes of the hybridoma supernatants for 1 hr at room temp., then added to the MCLR–BSA coated wells. After en overnight incubation et 4°C, HRP-SA diluted 1:10,000 in dilution buffer (PBS containing 0.5% (w/v) gelatin and 0.05% (v/v) Tween 20) was added and incubated for 2 hr at room temp. The enzyme reaction was started by adding the substrate solution (0.1 M sodium acetate buffer (pH 5.0) containing 100 μg/ml of TMBZ and 0.005% (v/v) H_2O_2) and stopped with 2 N H_2SO_4. The absorbance at 450 nm was measured with an ELISA reader (Dynatech Laboratories, Chantilly).

Estimation of specific binding of MAb-id to MAb-mc

(A) Indirect ELISA. Mutual binding between MAb-id and MAb-mc was examined by indirect ELISA. Microtiter plates were coated with MAb-mc or MAb-id (1 μg/ml in PBS), then blocked with blocking buffer. Various concentrations of biotinylated MAb-id (Bio-MAb-id) and biotinylated MAb-mc (Bio-MAb-mc) were respectively added to MAb-mc and MAb-id coated wells. The bound biotinylated MAbs (Bio-MAbs) were detected with HRP-SA as described in the screening ELISA. In some experiments, MCLR (100 ng/ml in blocking buffer) was added together with Bio-MAb-id or Bio-MAb-mc as a competitor.

(B) Immunoprecipitation. Bio-MAb-id or biotinylated control IgG_1 (Bio-IgG_1) (20 μg Ig) were reacted to MAb-mc-coupled gel or control IgG_1-coupled gel (equivalent to 1 mg Ig). After washing with PBS containing 0.05% (v/v) Tween 20, Bio-MAbs bound to the coupled gel were separated by 10% SDS-PAGE and transferred to Immobilon membrane (Millipore, Bedford). The blots were detected with ALP-SA followed by the BCIP/NBT phosphatase substrate system (Kirkegard and Perry Lab., Gaithersburg).

MAb-id based ELISA

MAb-id was tested for use in three competitive types of ELISA (type 1, 2 and 3).

(A) Type 1 (MAb-id was reacted with Bio-MAb-mc in the presence of MCLR). Microtiter plates were coated with MAb-id (1 μg/ml in PBS). Various concentrations of MCLR were mixed with an equal volume of Bio-MAb-mc (100 ng/ml in dilution buffer), then added to the coated wells. The bound Bio-MAb-mcs were detected with HRP-SAas described in the screening ELISA.

(B) Type 2 (MAb-mc was reacted with Bio-MAb-id in the presence of MCLR). Microtiter plates were coated with MAb-mc (1 μg/ml in PBS). Various concentrations of MCLR were mixed with an equal volume of Bio-MAb-id (40 ng/ml in dilution buffer), then added to the coated wells. All subsequent steps were as for type 1 ELISA.

(C) Type 3 (MAb-mc was reacted with MAb-id in the presence of MCLR followed by Bio-MAb-mc). Microtiter plates were coated with MAb-mc (2 μg/ml in PBS). Various concentrations of MCLR were mixed with an equal volume of MAb-id (60 ng/ml in dilution buffer), then added to the coated wells. The bound MAb-id was reacted with Bio-MAb-mc (1 μg/ml in dilution buffer). All subsequent steps were as for type 1 ELISA.

Comparison of MAb-id and MAb-mc based ELISAs

A comparative study of MAb-id based ELISA with previously established MAb-mc based ELISA was done with 25 environmental freshwater samples. All samples, collected from two lakes and one pond in Japan during June–September of 1996, had been stored at −30°C. The samples were freeze–thawed twice, filtered through a glass fiber filter, and then the contents of total MCs were directly assayed by MAb-id based ELISA (type 3) and MAb-mc based ELISA. All data were expressed as the mean of two determinations with MCLR as the standard.

RESULTS

Production of MAb-id

Two MAb-id (IgG_1, κ) producing hybridomas were selected based on their ability to inhibit the binding of MAb-mc to MCLR–BSA in ELISA The dose-dependent inhibition curves of the two MAbs (designated Id7 and Id8) are shown in Fig. 1(a). Both curves were parallel to that of MCLR, although the inhibitory activities of these MAbs were lower than that of MCLR. Concentrations causing 50% inhibition of the bindings (IC_{50}) were 2.80 nM, 22.4 nM, and 0.63 nM for Id7, Id8 and MCLR, respectively. Control IgG_1 did not affect the ELISA. Id7 was further characterized because of its higher inhibitory activity. Firstly, the mutual binding between Id7 and MAb-mc was examined in the ELISA. As shown in Fig. 1(b), biotinylated Id7 (Bio-Id7) bound to MAb-mc coated on the plates and reversely, Bio-MAb-mc bound to Id7 coated on the plates. Both bindings were dose-dependent. In addition, at concentrations of Bio-MAbs causing about 0.8 optical density (10 ng/ml for Bio-Id7, 100 ng/ml for Bio-MAb-mc), free MCLR (100 ng/ml) clearly inhibited their bindings (79% inhibition for Bio-Id7 and 53% inhibition for Bio-MAb-mc). Next, immunoprecipitation analysis was performed to examine the specific binding between Id7 and MAb-mc. Bio-Id7 or Bio-IgG_1 was reacted to MAb-mc-coupled or control IgG_1-coupled gel, then bound Bio-MAbs were analyzed by immunoblotting (Fig. 1(c)). As a result, Id7 reacted to MAb-mc-coupled gel but not to control IgG_1-coupled gel, while control IgG_1 did not react to both gels. The overall results indicated that Id7 and MAb-mc specifically interacted through the antigen binding sites of MAb-mc. Therefore, Id7 is a MAb-id to MAb-mc end possesses the structural image of MCLR.

The selection of the best format of MAb-id based ELISA

To develop a new ELISA using produced MAb-id, three competitive ELISAs were examined for their sensitivities and variations. The sensitivities of three types of MAb-id based ELISA were compared to that of MAb-mc based ELISA. The protocols of these ELISAs are shown in Fig. 2(a). The Bio-MAbs concentrations in three types of MAb-id based ELISA were selected to give about half-maximal absorbance at the points of the titration curves where linear binding occurred. Initially, Id7 was substituted for MCLR–BSA. In this ELISA (type 1), Id7 was coated on the plates, then reacted with Bio-MAb-mc. As shown in Fig. 2(b), this assay gave dose-dependent standard curve, however, the IC_{50} value was 20 ng/ml, which was over one hundred-fold less sensitive then that of MAb-mc based ELISA (IC_{50} = 70 pg/ml). To improve the assay sensitivity,

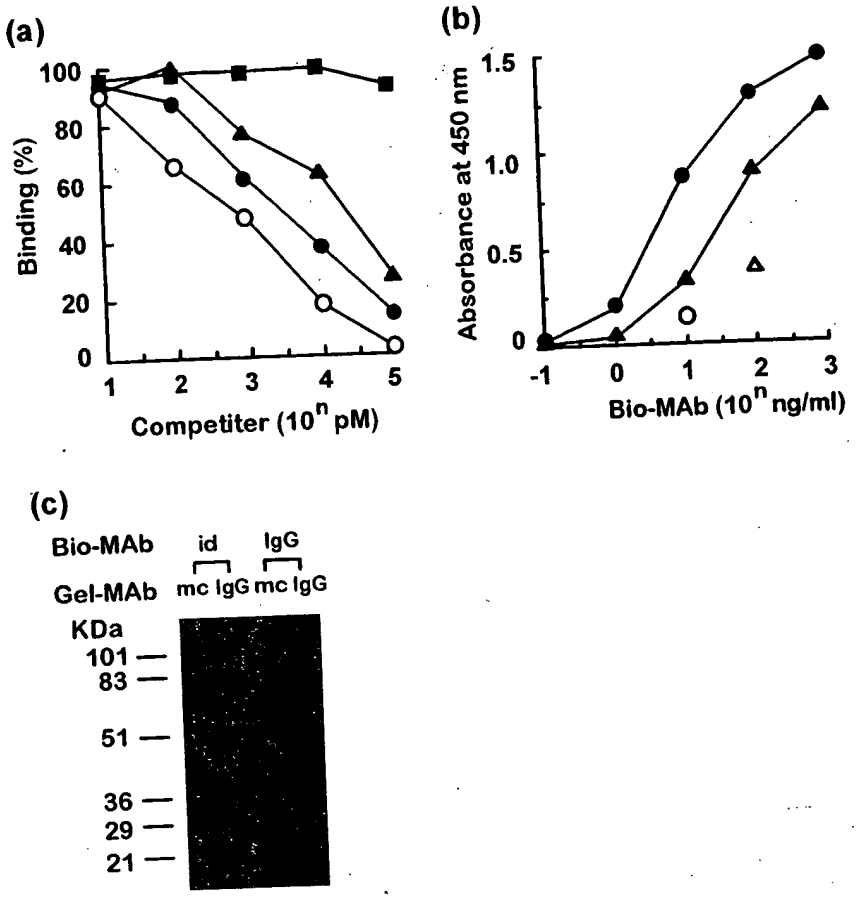

Fig. 1. Specific binding of MAb-id to the idiotype of MAb-mc.
(a) Inhibition of the binding of MAb-mc to MCLR–BSA by MAb-ids, control IgG_1 or MCLR in an ELISA. The microtiter plates were coated with MCLR–BSA (100 ng/ml), then reacted with biotinylated Fab fragments of MAb-mc (100 ng/ml) in the presence of various concentrations of Id7 (•), Id8 (▲), control IgG_1 (■) or MCLR (○). The biotinylated Fab fragments of MAb-mc bound to the coated MCLR–BSA were detected with HRP-SA as the second reagent. Resulting absorbances (450 nm) were expressed as the percentage of the absorbance without competitors.
(b) Mutual binding between MAb-id and MAb-mc in an indirect ELISA. The microtiter plates were coated with either MAb-mc or Id7 (1 μg/ml), then respectively incubated with various concentrations of Bio-Id7 (•) or Bio-MAb-mc (▲). The bound Bio-MAbs were detected with HRP-SA as the second reagent. Open symbols show the absorbances in the presence of MCLR (100 ng/ml).
(c) Immunoblots of Bio-MAb-id or Bio-IgG_1 reacted to MAb-mc-coupled gel or control IgG_1-coupled gel. Bio-Id7 or Bio-IgG_1 (20 μg Ig) were reacted to MAb-mc-coupled gel or control IgG_1-coupled gel (1 mg Ig equivalent). After washing, the bound Bio-MAbs were separated by SDS-PAGE and transferred to Immobilon membrane. The blots were detected with ALP-SA and BCIP/NBT substrate. The used Bio-MAbs and gel-coupled MAbs are shown at the top of the panel. The sizes of molecular weights standards are indicated in the left column.

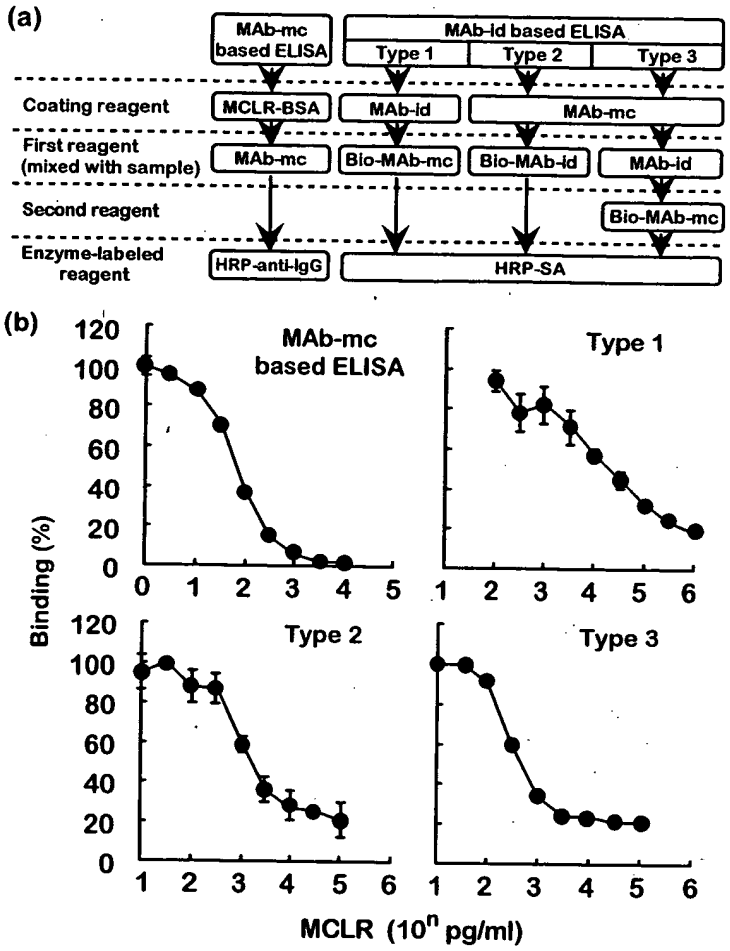

Fig. 2. Comparison of standard curves of three types of MAb-id based ELISA and previously established MAb-mc based ELISA.
(a) Protocols of three types of MAb-id based ELISA and MAb-mc based ELISA. The reagents used in each step are shown in boxes.
(b) Standard curves of three types of MAb-id based ELISA and MAb-mc based ELISA. Bars represents the means ± S.D. of three wells, which are missing when interfering with symbol.

a reverse assay, in which coated MAb-mc was reacted with Bio-Id7, was tested. In this ELISA (type 2), the IC_{50} value was 1.5 ng/ml, about a thirteen-fold improvement over the value for type 1 ELISA. Because of the higher sensitivity of type 2, we examined alternative biotinylated reagent for use in this formal. In the next ELISA (type 3), Bio-MAb-mc was used for the detection of Id7 bound to the coated MAb-mc. Since Id7 is bivalent, some proportion of Id7 can bind to Bio-MAb-mc. As a result, the IC_{50} value was 450 pg/ml, about a three-fold improvement over the value for the type 2 ELISA. This value was only a six-fold less sensitive than that of MAb-mc based ELISA. A typical sigmoidal curve of type 3 ELISA exhibited linearity within 100 to 1000 pg/ml of

MCLR with a coefficient of variation of less than 3%. Thus, type 3 ELISA was used in the following experiments because of its best performance.

Performance of MAb-id based ELISA for determination of MCs in environmental water

To test the practical use of MAb-id based ELISA for environmental monitoring, various MCs found in environmental water were examined for their cross-reactivities. Three MCs, MCRR, MCYR and 6(Z)-MCLR were tested in type 3 ELISA. As shown in Fig. 3, MCRR and MCYR, principal MCs as well as MCLR gave similar inhibition curves to MCLR. The IC_{50} values for MCRR and MCYR were 111% and 67% of MCLR, respectively. In contrast, 6(Z)-MCLR, the geometric isomer of Adda portion of MCLR and a minor component found in environmental water, was much weaker than MCLR (7% of MCLR). Therefore, the most of MCs found in environmental water could be almost equally measured by MAb-id based ELISA. As for the cross reactivity of MAb-mc based ELISA, the IC_{50} values were reported to 50, 80 and 790 pM for MCRR, MCYR and 6(Z)-MCLR, respectively (Nagata *et al.*, 1997). These values were corresponding to 126, 79 and 8% of MCLR, respectively, indicating the almost same cross reactivities between MAb-mc based ELISA and MAb-id based ELISA.

Next, to test the direct application of MAb-id based ELISA to environmental water, MCs concentrations in 25 freshwater samples were directly assayed by MAb-id based ELISA and compared with the results obtained by MAb-mc based ELISA. MAb-id based ELISA revealed that 14 of 25 samples were positive for MCs in the range from 130 to 1,300,000 pg/ml, while MAb-mc based ELISA revealed that 16 samples were

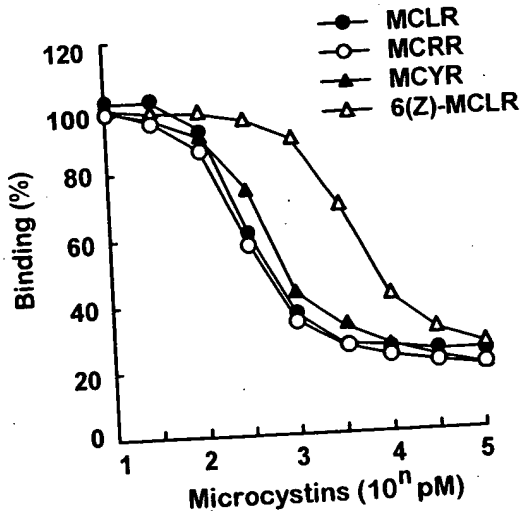

Fig. 3. The cross-reactivity of MAb-id based ELISA (type 3) against various microcystins. The microtiter plates were coated with MAb-mc (1 μg/ml), then incubated with the mixtures of Id7 (60 ng/ml) and various concentrations of MCLR (●), MCRR (○), MCYR (▲) or 6(Z)-MCLR (△). The Id7 bound to the coated MAb-mc possessed free antigen binding sites because of its bivalency. The free antigen binding sites of Id7 were detected with Bio-MAb-mc (1 μg/ml) followed by HPR-SA. Symbols represent the means of three wells. S.D. bars are missing because interfering with symbols.

Table 1. Comparison of microcystins content in environmental freshwaters as determined by MAb-id based ELISA (type 3) and MAb-mc based ELISA*

Sampling sites	Date	Microcystins (pg/ml)†	
		MAb-id based ELISA	MAb-mc based ELISA
Lake Inbanuma	06/13	ND‡	ND
	06/20	ND	ND
	06/27	Trace§	89
	07/04	130	120
	07/11	ND	ND
	07/25	400	420
	09/26	ND	97
Lake Kasumigaura	06/13	ND	ND
	06/20	ND	ND
	06/27	ND	ND
	07/04	ND	Trace
	07/11	ND	ND
	07/25	1,300,000 (250)‖	1,200,000 (240)
	08/01	17,000 (170)	16,000 (160)
	08/08	27,000 (270)	29,000 (290)
	08/15	140,000 (440)	130,000 (450)
	08/22	180,000 (360)	180,000 (350)
	08/29	76,000 (380)	72,000 (360)
	09/06	94,000 (470)	86,000 (430)
	09/19	340	310
	09/26	360	280
Pond Ushiku	06/17	ND	ND
	06/27	150	110
	07/11	380	390
	08/27	230	250

*All samples were collected in 1996. The samples preparation method allowed the complete extraction of cellular MCs into the water, so that total MCs (released MCs plus intracellular MCs) were measured (Nagata *et al.*, 1997). Some positive samples were diluted with distilled water containing 0.005% (w/v) gelatin to obtain the measurement range of the ELISAs.

†Values are mean of two determinations. Linear regression equation $y = 0.95x + 28.4$ ($r = 0.97$) was obtained for MAb-mc based ELISA vs MAb-id based ELISA. This equation was calculated with the measured values.

‡ND, not detected. Detection limit of MAb-id based ELISA and MAb-mc based ELISA is 100 and 50 pg/ml, respectively.

§Only one of the two determinations gave a significant value above the detection limits.

‖Values in parentheses indicate the measured MCs concentrations in the diluted samples.

positive in the range from 89 to 1,200,000 pg/ml (Table 1). The correlation coefficient was 0.97, which indicated a good agreement for these ELISAs. The slope of 0.95 and y-intercept of 28.4 were near to 1 and zero, respectively, suggesting that the environmental water matrix gave no significant interference to MAb-id based ELISA. Thus, MAb-id based ELISA worked well with the detection limits of 100 pg/ml of MCs in the natural water samples.

DISCUSSION

Anti-idiotype antibodies are generally classified into three types, α, β and γ according to their specificities for different regions of the primary antibody (Bona and Kohler, 1984). Type α binds to the idiotype outside the antigen binding sites, therefore it does not inhibit the binding of the primary antibody to the antigen. In contrast, type β and γ bind to the idiotype associated with the antigen binding sites,thereby inhibiting the

binding of the primary antibody to the antigen. Type β, possesses the internal image of antigen so that it sometimes displays biological activities similar to the antigen. Type γ potentially binds to the idiotype located close to the antigen binding sites of the primary antibody and interferes with the binding of the antigen through steric hindrance.

Our screening assay was designed to detect type β or γ of MAb-id that could inhibit the binding of MAb-mc to MCLR. If Id7 is classified into type β, it is expected to be applied for studies in toxicology (Nisonoff, 1991). Several type β of MAb-ids have served as research tools, such as probes for the receptors of the antigens. Also, type β can induce immune-mediated responses similar to those induced by the original antigen, and this property has been used to produce vaccines (Chanh *et al.*, 1992). Since it has been found that MCs bind to PP1 and PP2A and inhibit their activities (Honkanen *et al.*, 1990; Yoshizawa *et al.*, 1990), we examined whether Id7 could inhibit PP1 and PP2A activities. In addition, we examined whether immunization of Id7 could induce antibody against MCLR. Regrettably, these limited attempts failed to reveal biological mimicry between Id7 and MCLR (data not shown). The findings may suggest that Id7 is classified as a type γ.

This is the first report on development of MAb-id based ELISA for MCs. To date, over 50 MCs have been identified and most of them exhibit toxicity. ELISA for monitoring should detect most of them (Nagata *et al.*, 1997). The present MAb-id based ELISA probably detected most of toxic MCs, because the cross-reactivity data (Fig. 3) suggests that MAb-id based ELISA recognized the tertial structure of Adda, which is a commonly shared and important structure for toxic MCs (Harada *et al.*, 1990; Nishiwaki-Matsushima *et al.*, 1991; Stotts *et al.*, 1993). This wide cross-reactivity was similar to that of MAb-mc based ELISA (Nagata *et al.*, 1997). Since both ELISAs showed a very similar pattern of the cross-reactivities against principal MCs found in environmental water, total MCs in environmental water determined by MAb-id based ELISA were probably agreed with the values obtained by MAb-mc based ELISA. In addition, in the analysis of freshwater samples, MAb-id based ELISA gave almost the same results as MAb-mc based ELISA (Table 1). These results showed that MAb-id based ELISA exhibits the almost same performance as MAb-mc based ELISA for direct determination of MCs in freshwater, and is suitable for monitoring of MCs. Addition to this, there is one potential advantage in MAb-id based ELISA. In MAb-mc based ELISA, lot-to-lot variations of MCLR–BSA used for coating is a factor affecting assay reproducibility. MAb-id based ELISA eliminates this possible drawback by excluding MCLR–BSA from this assay.

In conclusion, we produced MAb-id against MAb-mc, and developed a new ELISA. Thus, MAb-id based ELISA represents a useful alternative for monitoring MCs levels in the environments.

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Mary E. (Molly) Ceperley
Primary Examiner Art Unit 1641
(703) 308-4239
Office: CM1-8D15
Mailbox: CM1-7E12

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Detection and Identification of Metabolites of Microcystins Formed *in Vivo* in Mouse and Rat Livers

Fumio Kondo,*,† Hiroshi Matsumoto,† Seiji Yamada,† Naohisa Ishikawa,‡ Emiko Ito,‡ Satoshi Nagata,§ Yoshio Ueno,§ Makoto Suzuki,|| and Ken-ichi Harada||

Aichi Prefectural Institute of Public Health, Tsuji-machi, Kita, Nagoya 462, Japan, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Inohana, Chuo, Chiba 260, Japan, Faculty of Pharmaceutical Sciences, Science University of Tokyo, Ichigaya, Tokyo 162, Japan, and Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468, Japan

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The hepatic metabolism of microcystins (MCs), potent cyclic peptide hepatotoxins produced by cyanobacteria, was studied by ip injection in mice and rats. An immunoaffinity purification method using an anti-MC-LR monoclonal antibody showed a remarkable effect on the removal of contaminants in the hepatic cytosol and enabled us to analyze MCs and their metabolites by HPLC and Frit-FAB LC/MS. At 3, 6, and 24 h post-injection of MC-RR, a small percent of the applied dose was detected in all of the mouse livers together with several metabolites. Among them, GSH and Cys conjugates of MC-RR were identified at 3 and 24 h, respectively, by comparison with the chemically prepared standards, indicating that the thiols of GSH and Cys nucleophilically bound to the Mdha moiety of MCs. Another metabolite was presumed to be formed by both epoxidation followed by hydrolysis and sulfate conjugation in the Adda moiety and GSH conjugation in the Mdha moiety. In rat livers, MC-LR showed almost the same behavior as that of MC-RR in mouse livers. These results suggest that the conjugation of GSH with MCs may play a role in the metabolic pathway leading to detoxification of MCs.

Introduction

Microcystins (MCs),[1] cyclic hepatotoxic heptapeptides produced by cyanobacteria, have a common array of five amino acids, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), *N*-methyldehydroalanine (Mdha), D-alanine, β-linked D-*erythro*-β-methylaspartic acid, and γ-linked D-glutamic acid, and two L-amino acids as variants (*1*). MCs cause poisonings of wild and domestic animals and also represent a public health threat to humans through drinking water (*1*). It has been found that MCs are potent inhibitors of protein phosphatases 1 and 2A and have a tumor-promoting activity in the rat liver (*2*−*6*). MacKintosh et al. (*7*) and Runnegar et al. (*8*) reported that MCs bind covalently to cysteine-273 and -266 on protein phosphatase 1 and 2A, respectively.

In several reports, the tissue distribution, excretion, and hepatic metabolism of [^{3}H]MC-LR and [^{3}H]dihydroMC-LR have been studied (*9*−*11*). In the liver, a rapid uptake of radiolabeled MCs occurred when intraperitoneally or intravenously injected, remaining for at least 6 days (*9, 10*), whereas po administration of [^{3}H]-dihydroMC-LR showed a much lower uptake into the liver (*10*). There have been conflicting reports concerning the hepatic metabolism of MCs; Robinson et al. documented putative detoxification products by iv injection of [^{3}H]MC-LR into mice (*9*), while Rinehart et al. described that [^{3}H]dihydroMC-LR injected intraperitoneally to pigs seemed not to be highly metabolized (*11*).

In previous reports using radiolabeled MCs, the amounts of injected MCs were too small and those of contaminants in tissues were too large to investigate the metabolites by instrumental analysis, such as HPLC and MS. For this reason, more effective cleanup and sensitive determination methods were required for such an investigation. In the present study, we introduced an immunoaffinity purification method using an anti-MC-LR monoclonal antibody (MAb) that can selectively recognize MCs (*12*), resulting in a remarkable effect on the removal of contaminants in the hepatic cytosol. After the immunoaffinity purification, MCs and their metabolites were analyzed by HPLC and Frit-FAB LC/MS (*13, 14*). Here we describe the detection and identification of metabolites of MC-RR and MC-LR formed *in vivo* in mouse and rat livers, respectively.

Experimental Procedures

Caution: *MCs are hazardous due to their potent hepatotoxicity and tumor-promoting activity and should be handled carefully.*

Isolation of MC-RR and MC-LR. MC-RR and MC-LR (Figure 1) were isolated and purified from surface blooms collected from Lake Suwa in Japan, as described previously (*15*).

Animals. Male, 5-week-old ddY mice (Nippon SLC, Hamamatsu, Japan) and male 5-week-old Wister rats (Charles River Japan, Atsugi, Japan) were used.

Preparation of Immunoaffinity Column. Affi-Gel-10 (25 mL, Bio-Rad, Hercules, CA) was washed with iced water (250 mL) and mixed with an equal volume of 10 mg/mL M8H5 anti-MC-LR MAb in phosphate-buffered saline (PBS) (pH 7.4) (*12*).

* Correspondence should be addressed to this author at the Aichi Prefectural Institute of Public Health, Tsuji-machi, Kita, Nagoya 462, Japan. Phone 81-52-911-3111 ext 251; FAX 81-52-913-3641.
† Aichi Prefectural Institute of Public Health.
‡ Chiba University.
§ Science University of Tokyo.
|| Meijo University.
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[1] Abbreviations: MC(s), microcystin(s); Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Mdha, *N*-methyldehydroalanine; MAb, monoclonal antibody; ODS, octadecyl silanized; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid.

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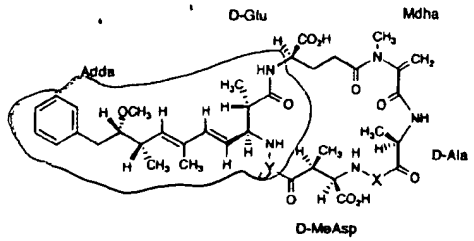

	X	Y
Microcystin-RR	L-Arg	L-Arg
Microcystin-LR	L-Leu	L-Arg

Figure 1. Structures of microcystins-RR and -LR.

The gel mixture was incubated at 4 °C for 24 h with gentle rocking. After the addition of 1 M ethanolamine hydrochloride (2.5 mL), the gel mixture was washed with water (250 mL) followed by PBS (500 mL). Obtained gel cake was suspended in PBS containing 0.1% sodium azide (50 mL) and stored at 4 °C. The gel mixture (1 mL) was transferred to Muromac columns (Muromachi Kagaku Kogyo Kaisha Ltd., Tokyo, Japan) and was washed with PBS (10 mL), methanol (10 mL), water (10 mL), and PBS (10 mL) prior to use.

Preparation of Cytosolic Extracts from Mouse and Rat Livers. Mice were injected ip with 10 or 20 μg/head MC-RR. At 3, 6, and 24 h post-injection, the livers were removed and weighed. Isolated mouse livers were minced in 100 mM Tris-HCl buffer (pH 7.2, 5 mL), homogenized, and centrifuged at 10000*g* for 10 min. The operation was repeated, and the resulting supernatant was combined and then centrifuged at 100000*g* for 60 min. The supernatant was heat denatured for 30 min at 90 °C after addition of an equal volume of 100 mM potassium phosphate buffer (pH 7.5). Then the solution was treated with Pronase (20 mg, Boehringer Mannheim Biochemicals, Indianapolis, IN) and incubated for 60 min at 37 °C. After three cycles of heat denaturation and Pronase digestion, the resulting solution was centrifuged at 10000*g* for 10 min, and the supernatant was applied to a Sep-Pak octadecyl silanized (ODS) cartridge (1 g, Millipore Co., Milford, MA). The cartridge was washed with water (20 mL), and the eluate from the cartridge with 90% aqueous methanol (30 mL) was evaporated to dryness. The resulting residue was dissolved in PBS (10 mL), and the solution was subjected to an immunoaffinity column. After washing with PBS (10 mL), followed by water (10 mL), the desired fraction was eluted with methanol (10 mL). The eluate was evaporated to dryness, and the residue was subjected to HPLC and Frit-FAB LC/MS analysis.

Rats were injected ip with 4 μg/head MC-LR. At 24 h post-injection, the livers were removed and weighed. Isolated rat livers were treated as described above.

HPLC and Frit-FAB LC/MS Conditions. For HPLC analysis, a Shimadzu (Kyoto, Japan) LC-6A pump coupled to an SPD-6AV set at 238 nm and a C-R6A integrator was used. Separation was accomplished under reversed phase isocratic conditions with a Nucleosil 5C18 column (5 μm, 4.6 × 150 mm, Chemco Scientific Co., Osaka, Japan) and a mobile phase of acetonitrile/0.02 M ammonium acetate (23:77 or 21:79). The flow rate was 1.0 mL/min. For the UV spectral measurements of the observed peaks on the HPLC chromatogram, a Model 991J HPLC photodiode array detector system (Millipore Co.) was used. Separation was accomplished under reversed phase isocratic conditions with a Nucleosil 5C18 column (5 μm, 4.6 × 150 mm) and a mobile phase of methanol/0.05% trifluoroacetic acid (TFA) (45:55). The flow rate was 0.8 mL/min.

For Frit-FAB LC/MS analysis, an LC-100P HPLC pump (Yokogawa Electric, Tokyo, Japan) coupled to an SPD-2A (Shimadzu) set at 238 nm was connected to a Frit-FAB interface. Separation was achieved using a Develosil ODS-HG-5 column (150 × 0.3 mm, Nomura Chemical, Seto, Japan) and a mobile phase of methanol/0.05% TFA (56:44 or 55:45) containing 0.8% glycerol. The solvent flow was split between the HPLC pump and the Rheodyne (Cotati, CA) Model 7125 injector using a restriction column, so that 4 μL/min was introduced into the mass spectrometer through the UV detector. A mass spectrometer (JMS-AX505W, JEOL Co., Tokyo, Japan) was used and connected to a data system JEOL JMA-DA5000. The FAB MS data (resolution 1500) were obtained in the positive-ion mode by scanning from *m/z* 50 to *m/z* 1500 with a scan speed of 7.5 s/cycle and were corrected to give nominal mass values by subtracting proportionally at the ratio of 0.7 amu/1000 amu. A FAB gun was operated at 3 kV with xenon gas, and the spectrometer was operated at a 5 kV accelerating potential.

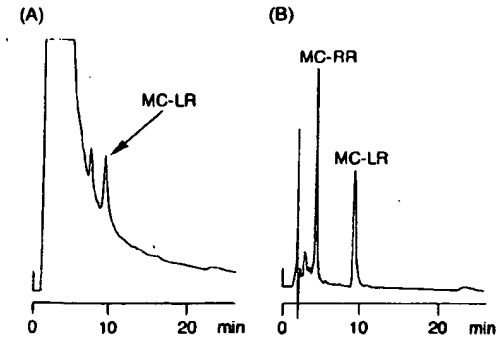

Figure 2. HPLC profiles of a cytosolic extract from mouse liver spiked with 5 μg each of MC-RR and MC-LR. Shown are (A) an extract after heat denaturation, pronase digestion, and ODS silica gel cleanup and (B) further immunoaffinity purification. Column: Nucleosil 5C18, 150 × 4.6 mm. Mobile phase: methanol/0.05% TFA (56:44). Flow rate: 0.8 mL/min. Detection: 238 nm.

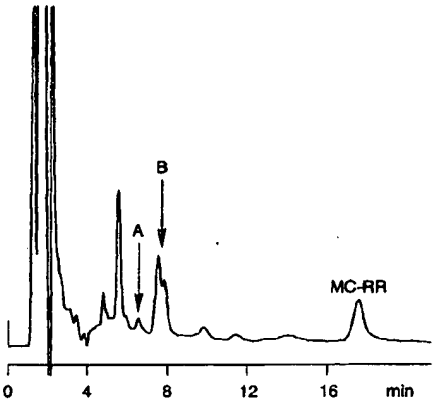

Figure 3. HPLC profile of a cytosolic extract from mouse liver at 3 h post-injection of MC-RR (20 μg/head). Column: Nucleosil 5C18, 150 × 4.6 mm. Mobile phase: acetonitrile/0.02 M ammonium acetate (23:77). Flow rate: 1 mL/min. Detection: 238 nm.

Results and Discussion

Immunoaffinity Purification. As indicated by the previous study, a large number of contaminants in the hepatic cytosol interfered with the mass spectrometric analysis of MCs and their metabolites (*11*). In fact, when the hepatic cytosol (spiked with 5 μg each of MC-RR and MC-LR) was prepared for HPLC with UV detection (238 nm) by the same treatment as described by Robinson et al., which consisted of heat denaturation, Pronase digestion, and ODS silica gel cleanup (*9*), MC-RR and MC-LR could not be precisely analyzed (Figure 2A), indicating that a more effective cleanup method was required. We initially used a cleanup method utilizing the a combination of ODS silica gel and silica gel cartridges, which has been successfully applied to analyze trace amounts of

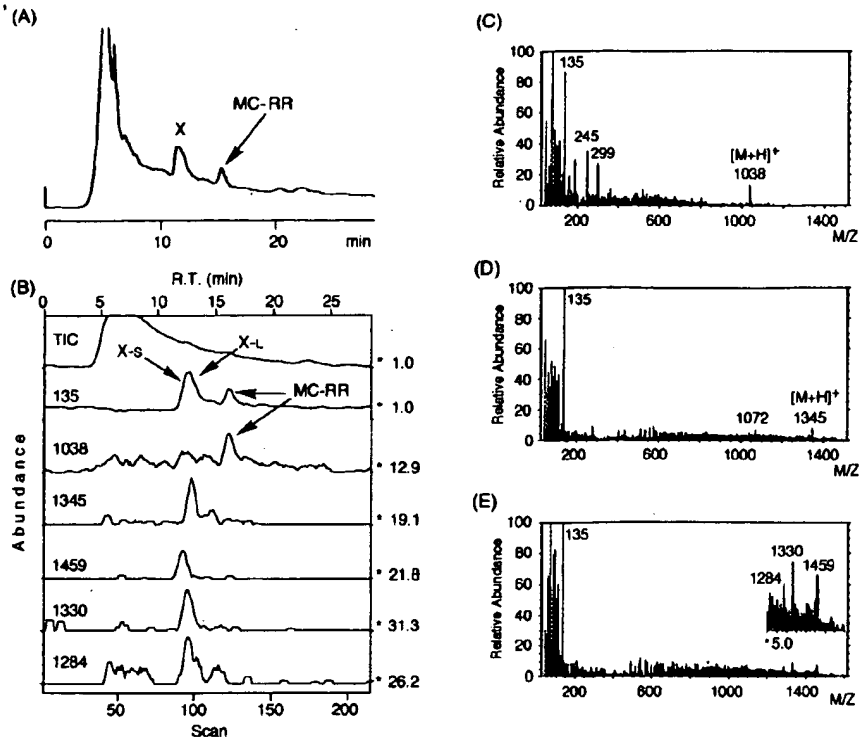

Figure 4. Frit-FAB LC/MS analysis of a cytosolic extract from mouse liver at 3 h post-injection of MC-RR (20 μg/head). Shown are (A) simultaneously monitored UV chromatogram (238 nm), (B) total ion and mass chromatograms monitored at m/z 135, 1038, 1345, 1459, 1330, and 1284, (C) Frit-FAB LC/MS mass spectra of MC-RR, (D) longer retention time area of the peak X (indicated by X-L), and (E) shorter retention time area of the peak X (indicated by X-S). Frit-FAB LC/MS conditions are as follows: HPLC, column, Develosil ODS-HG-5, 150 × 0.3 mm; mobile phase, methanol/0.05% TFA (55:45) containing 0.8% glycerol; flow rate, 4 μL/min. MS, ion source, Frit-FAB; primary beam, Xe^0 (5 kV); scan range, m/z 50–1500.

MCs in lake water (*14*, *16*). However, it was insufficient to eliminate contaminants in the hepatic cytosol (data not shown). Thus we introduced an immunoaffinity purification using an anti-MC-LR MAb that selectively recognized both MC-RR and MC-LR (*12*). After the immunoaffinity purification of the cytosolic extract prepared above, the contaminants were effectively eliminated and the peaks of MC-RR and MC-LR were clearly detected (Figure 2B). Additionally, the overall recovery from extraction to immunoaffinity purification was almost the same (approximately 65%) as that reported by Pace et al. (*17*). In the following experiments, hepatic cytosols of mice and rats dosed with MCs were prepared for HPLC and Frit-FAB LC/MS analysis by a procedure composed of heat denaturation, Pronase digestion, ODS silica gel cleanup, and immunoaffinity purification.

Analysis of MC-RR and Its Metabolites in Mouse Livers. The HPLC profile (238 nm) of a cytosolic extract from mouse liver at 3 h post-injection of MC-RR (20 μg/head) shows a peak of MC-RR (Figure 3). At 3, 6, and 24 h post-injection, a small percent of the injected MC-RR was detected in the livers, and this result was almost consistent with that reported by Robinson et al. (*9*). The HPLC profile also shows peaks A and B having shorter retention times compared to that of MC-RR (Figure 3), both not found in the control hepatic cytosol extract. Robinson et al. reported putative detoxification products which also had shorter retention times compared to that of [^{3}H]MC-LR under almost the same HPLC conditions used in the present experiment (*9*).

To characterize them structurally, Frit-FAB LC/MS analysis was carried out (Figure 4A), in which the

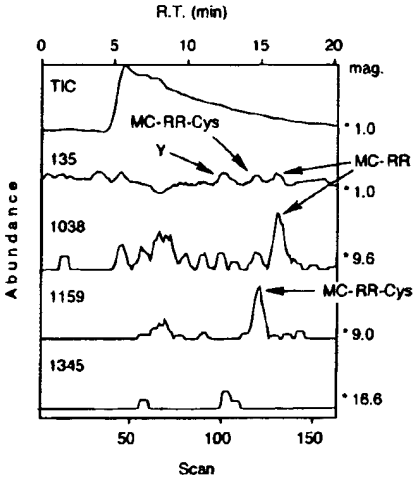

Figure 5. Frit-FAB LC/MS analysis of a cytosolic extract from mouse liver at 24 h post-injection of MC-RR (10 μg/head). Shown are total ion and mass chromatograms monitored at m/z 135, 1038, 1159, and 1345. Frit-FAB LC/MS conditions were described in Figure 4.

simultaneously monitored UV chromatogram (238 nm) shows the difference in separation from that of HPLC analysis (Figure 3), because the HPLC conditions used in Frit-FAB LC/MS were optimized for the analysis of MCs (*13*, *14*), while those used in HPLC analysis were intended to be almost identical to those used by Robinson et al. (*9*). The mass chromatography monitored at the ion at m/z 135, which is formed by α-cleavage of a methoxy group of the Adda moiety in MCs under FAB

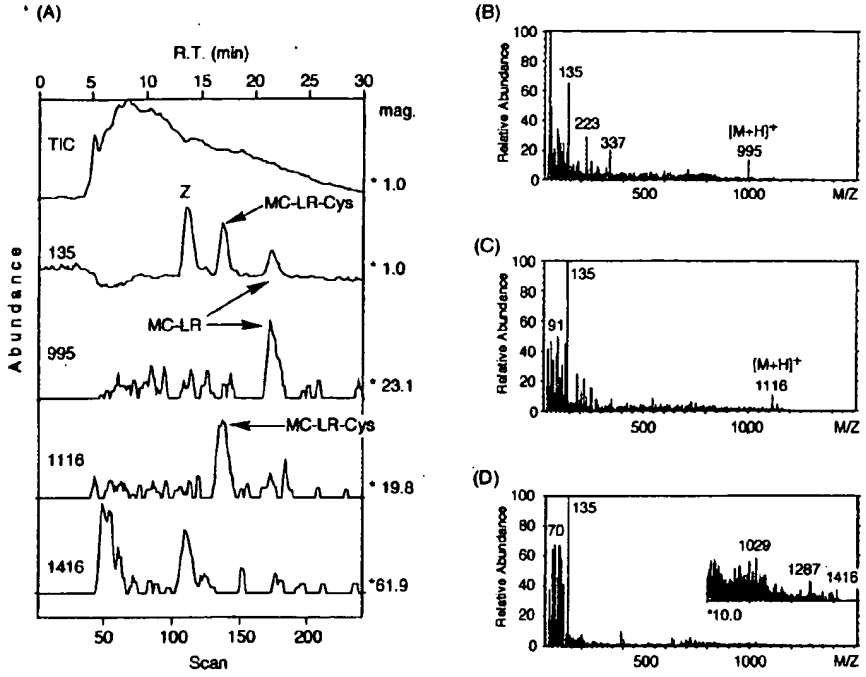

Figure 6. Frit-FAB LC/MS analysis of a cytosolic extract from rat liver at 24 h post-injection of MC-LR (4 μg/head). Shown are (A) total ion and mass chromatograms monitored at *m/z* 135, 995, 1116, and 1416, (B) Frit-FAB LC/MS mass spectra of MC-LR, (C) Cys conjugate of MC-LR, and (D) peak Z. Frit-FAB LC/MS conditions are as follows: mobile phase, methanol/0.05% TFA (56:44) containing 0.8% glycerol. The other conditions were described in Figure 4.

conditions, is a powerful technique for discriminating MCs and related compounds from other types of compounds (*13, 14, 18, 19*). It shows a relatively broad peak X, which contained at least three peaks as will be described later, was detected together with intact MC-RR (Figure 4B). The mass spectrum of the longer retention time area of the peak X showed an ion at *m/z* 1345, whose molecular weight tentatively corresponded to that of the GSH conjugate of MC-RR (Figure 4D), the thiol of GSH having added nucleophilically to the α,β-unsaturated carbonyl of the Mdha moiety in MCs as reported previously (*20*). Peak A detected in the HPLC analysis of the hepatic cytosol extract showed the same retention time as that of the chemically prepared GSH conjugate of MC-RR, affording the confirmation (Figure 3).

The mass spectrum of the shorter retention time area of the peak X showed ions at *m/z* 1284, 1330, and 1459 with relatively low intensities (Figure 4E), and the mass chromatograms monitored at the ions provided the respective peaks with almost the same retention times (Figure 4B). HPLC analysis using slightly modified conditions revealed that peak X contained at least three peaks in addition to that of the GSH conjugate of MC-RR, one of which showed no typical absorption maximum of MCs at 238 nm. These results suggested that the shorter retention time area of peak X contained a compound whose structure is modified at the conjugated diene of the Adda moiety. It is generally supported that epoxidation, a phase I reaction followed by the conjugation as a phase II, plays an important role in hepatic metabolism of xenobiotics. Therefore, speculatively, the compound possessing a molecular weight of 1458 seemed to be formed by both epoxidation followed by hydrolysis and sulfate conjugation in the Adda moiety and GSH conjugation in the Mdha moiety. The ion at *m/z* 1330 differed from that at *m/z* 1459 by 129 amu, whose *m/z* value corresponded to the loss of the glutamic acid moiety from the compound with a molecular weight of 1458. No structural information about the ion at *m/z* 1284 has been obtained. In order to identify them, studies on chemical preparation of these compounds and on substrate specificity of the MAb with derivatives at the Adda moiety are just in progress.

Frit-FAB LC/MS analysis data for a cytosolic extract from mouse liver removed at 24 h post-injection of MC-RR (10 μg/head) are shown in Figure 5. The mass chromatogram at *m/z* 135 showed two peaks in addition to that of MC-RR, one of which was identified as the Cys conjugate of MC-RR by the comparison of its retention time with that of a chemically prepared standard. Information concerning the metabolites of MCs has been previously provided by only Robinson et al., who prepared the cytosolic extract with repeated Pronase digestion and used radiolabeled MC-LR for detecting the metabolites. The particular preparation method inherently causes the ambiguity that it is impossible to distinguish between GSH (*21*) and phosphatase adducts (*7, 8*) as the source of the Cys conjugates of MCs. We are now trying to develop a method that enables us to differentiate them. We have no structural information on peak Y because of very limited sample size; however, it might be a compound similar to the shorter retention time area of peak X shown in Figure 4.

Analysis of MC-LR and Its Metabolites in Rat Livers. As described before, the amounts of recovered MC-RR and its metabolites in mouse hepatic cytosol were almost the detection limit level for Frit-FAB LC/MS analysis, even though the LD_{50} level of MC-RR was injected. The hepatic metabolism of MC-LR, whose toxicity is approximately 10 times greater than that of MC-RR, was investigated by using rats instead of mice. Frit-FAB LC/MS analysis data for a cytosolic extract from rat liver removed at 24 h post-injection of MC-LR (4 μg/

head) are shown in Figure 6. The mass chromatogram at m/z 135 showed a peak of MC-LR and two peaks, one of which was identified as the Cys conjugate of MC-LR by the comparison of its retention time with that of the chemically prepared standard (*20*). The mass spectrum of peak Z did not provide any structural information; however, the faintly observed ion at m/z 1416 may correspond to the ion at m/z 1459 as observed for MC-RR, because the difference in their m/z values was consistent with that of the molecular weight of MC-RR and MC-LR. Additionally, the mass chromatogram monitored at m/z 1416 shows the corresponding peak, affording further evidence.

In the present study, we introduced the immunoaffinity purification method using an anti-MC-LR MAb for the investigation of the hepatic metabolism of MCs instead of using radiolabeled materials, which enabled us to analyze MCs and their metabolites by HPLC and Frit-FAB LC/MS. The GSH and Cys conjugates of MCs were identified, and a metabolite structurally modified in both the Adda and Mdha moieties has been proposed. It is conceivable that the conjugation with GSH would be a key reaction in the metabolism of MCs, although the Mdha moiety was not important for the toxic effects of MCs as indicated in our previous report (*20*) and in other studies (*7, 8*). Additionally, because MAb used in the present study showed variable cross-reactivity with various MCs and related compounds (*12*), other metabolites may possibly be formed in the liver, requiring further investigation.

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